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PREPARED FOR: U.S. Army Medical Research and Materiel Command
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Introduction: The DA award entitled “Elucidating Metastatic Behavior Through Nanopatterned Membranes” proposed to study the behavior of live human breast cancer cells (out of a library of over 60 cell lines) on artificial surfaces that mimic the microenvironment encountered in normal tissue. Natively membrane-bound ephrin-A1, which activates the cancer-associated receptor tyrosine kinase (RTK) EphA2 (1,2), is tethered to a two-dimensionally fluid supported membrane. Live human breast cancer cells expressing EphA2 are then engaged with these surfaces, and subsequent changes in receptor spatial organization, signaling, and cell behavior are quantified. Finally, the response of cells to various drugs can be measured to find effective treatments that change diseased-cell behavior. This approach may provide for cost effective, rapid, single-cell diagnostic assays that can better match therapeutics to patients. The use of supported membranes to study cancer behavior at the single cell level is an entirely new avenue for breast cancer diagnostics and if successful will provide for an assay that complements existing biomarker based diagnostics.

Body: As described in Aim 1, Task 1 of the Statement of Work, we have successfully cultured live human breast cancer cells on supported lipid bilayers functionalized with ephrin-A1, and imaged these surfaces using both epifluorescence and total internal reflection fluorescence microscopy (Appendix A, Figures 1, 2, 4). We have further analyzed the dynamics of ephrin-A1 cluster formation and transport using real time fluorescence imaging of these surfaces (Appendix A, Figures 2B, S4), as proposed in Aim 1, Task 2. Finally, as described in Aim 1, Task 3, we have fabricated chromium diffusion barriers onto these substrates and observed the effect of these mechanical perturbations to receptor transport on downstream signaling cascades. (Appendix A, Figure 4). In particular, we have seen a change in cytoskeleton morphology and recruitment of the metalloprotease ADAM10 (3,4) based on the geometry of these nanofabricated diffusion barriers.

Concerning Aim 2, Task 1, we have performed western blots to determine the degree of EphA2 activation as a function of ephrin-A1 presentation on the supported lipid bilayer (Appendix A, Figure 2F). EphA2 activation was quantified by measuring receptor phosphorylation and degradation, two known hallmarks of EphA2 activation (5). We also examined mRNA and protein expression across a library of 26 human mammary epithelial cell lines and discovered a panel of 37 proteins and 141 mRNAs that serve as surrogate biomarkers for EphA2 radial transport. Of these 37 proteins, 3 have thus far been validated as being spatially reorganized upon ephrin-A1 stimulation, including the receptor-ligand pair itself and CD44 (Appendix A, Figures S12-S13).

There are several candidate drugs targeting the actin cytoskeleton and microtubule formation that may be used to accomplish Tasks 2 and 3 of Aim 2. However drugs specifically targeting EphA2 have not been identified. We have requested a 12-month no-cost extension for this award (Appendix B) to identify drugs that are expected to alter EphA2 receptor transport and subsequent signaling processes. Then, using the platform developed herein, we can determine if any of these therapeutics would potentially serve a subset of breast cancer patients with greater efficacy by altering EphA2 receptor transport. This would allow for the proposed goal of personalized cancer therapy.

Key Research Accomplishments:

During the no-cost extension of this award into the second year, which was the result of administrative delays in distribution of the funds to Berkeley, the following activities occurred. The primary goal of this period was to complete the project and publish the results. At the time of the first 'final' report, which was submitted at the end of the first year period, we had a manuscript submitted to Science and were working on follow-up experiments concerning the effects of cytoskeleton modulating drugs. In response to reviewer comments on the work, we redoubled efforts specifically on the effects of a Rho-kinase inhibitor. These new results demonstrated that the process we have been observing can indeed be modulated by small molecule drugs.

These new results were also added to the manuscript, which was ultimately accepted for publication in Science. So, it is important to note that the publication resulting from this project that was appended in the first year report as a submitted manuscript was published in the second year no-cost extension period and that the published manuscript is not the same as the earlier submitted version. Key experiments performed with the remaining funds from this award are included in the final publication. This was not called out specifically in our previous submission of the report, and it would have required an actual reading of the manuscript, which was appended to the report, to see what were the new experiments. I apologize for any confusion this may have created.

Reportable Outcomes:

1. 1 published paper, 2010
2. 1 patent application submitted, 2009
3. 4 oral presentations, 2009, 2010
4. 2 poster presentations, 2009
5. Provided partial funds for 1 Ph.D. candidate, 2008-09.
6. Submitted application for US DOD BCRP Idea Award, 2009 and a modified proposal in 2010, which has been recommended for funding.

Conclusion: The completed research first yielded surprising discoveries in the role of receptor organization in EphA2-ephrin-A1 signaling in breast cancer. Since there is a strong correlation between EphA2 expression and tumor invasion *in vivo*, we next correlated the newly identified receptor organization phenotype against the likelihood that cells would invade foreign tissue across a library of human mammary epithelial cell lines. The strong correlation between these two cell behaviors (stronger even than the relationship between EphA2 protein or mRNA expression and invasion potential), demonstrates that the spatial organization component signaling allowed by the supported lipid bilayer platform is critical to the activity of ephrin-A1. Finally, using nanofabricated substrates that allow for precise control of allowed diffusion on the submicron length scale, we were able to discern an increase in recruitment of the metalloprotease ADAM10 as EphA2 radial transport was allowed to a greater degree. This change in protein recruitment was accompanied by a change in cytoskeleton morphology. Together, these data suggest a model whereby invasive cells radially transport EphA2, recruit ADAM10 protease to shed ephrin-A1 presented on apposing cell membranes, and thereby escape normal ephrin-mediated control of cell adhesion and repulsion. This result will allow for the development and screening of therapeutics whose mechanism of action is to alter EphA2 spatial organization, thereby altering downstream signaling cascades responsible for tumor invasion.

1. Publications:

Science, 2010, 327, 1380: "Restriction of receptor movement alters cellular response: physical force sensing by EphA2", Khalid Salaita*, Pradeep M. Nair*, Rebecca S. Petit, Richard M. Neve, Debopriya Das, Joe W. Gray, and Jay T. Groves.

2. Patent Applications:

A. Salaita, K., Nair, P. M., Das, D., Gray, J. W., Groves, J. T. Live Cell Activation Assay, US Patent Application 61/148,203, 2009.

3. Oral Presentations:

A. Nair, P. M., Eph-Ephrin Signaling in Breast Cancer: A hemomechanical Switch," University of California, Berkeley Biophysics Retreat, September 25-27, 2009.

B. Salaita, K., Groves, J. T., Deconstructing Receptor Signaling with Chemically Nanopatterned Interfaces, Nano Science and Technology Institute, International Conference on Nanofabrication Technologies, Devices and Applications, May 3-7, 2009, Houston, TX.

4. Poster Presentations:

- A. Nair, P. M., Salaita, K., Neve, R. M., Das, D., Gray, J. W., Groves, J. T., Role of Spatial Organization in Receptor Function: Eph-Ephrin Signaling in Breast Cancer," Poster. European Science Foundation and European Molecular
- B. Biology Organization Symposium, Biological Surfaces and Interfaces, June 27-July 2, 2009, Sant Feliu de Guixols, Spain.

Abstract: Activation of the EphA2 receptor tyrosine kinase by its natively membrane-bound ligand, ephrin A1, plays an important role in breast cancer biology. In vivo receptor-ligand binding is followed by EphA2-ephrin A1 complex formation, activation of the receptor, and ultimately receptor endocytosis and degradation. These events are all recapitulated when EphA2-expressing cells human breast cancer cells are cultured on a supported lipid bilayer (SLB) presenting laterally mobile, membrane-tethered ephrin A1. However ligand-induced receptor clustering and activity is altered when the ligand is presented in solution or bound to solid surfaces. Real-time fluorescence imaging, immunostaining, and protein expression analyses all show that the membrane-presented ligand induces a large-scale receptor spatial reorganization phenotype. Moreover, this phenotype can be shown to correlate with disease characteristics. Using nanopatterned substrates to impose 7 geometrically defined barriers to lateral mobility, it is possible to restrict and guide the assembly of these patterns in living cells. We refer to this technique as a spatial mutation. Our results indicate that mechanical restriction of EphA2 pattern formation can alter the outcome of its signaling pathway, which may also have consequences for certain aspect of cancer metastasis.

B. Nair, P. M., Salaita, K., Neve, R. M., Das, D., Gray, J. W., Groves, J. T.,
Spatially Regulated Ephrin A1-EphA2 Signaling in Breast Cancer,“ Poster award (3rd place).
International Society for Pharmaceutical Engineering, San Francisco Bay Area Chapter Student Poster Competition, May 9, 2009, Foster City, CA.

Abstract: Overexpression of the EphA2 receptor tyrosine kinase is observed in 40% of human breast cancers and receptor overexpression is sufficient to induce tumorigenesis in mammary epithelial cells. EphA2 activation by its membrane-bound ephrin A1 ligand occurs at the junction between two cell membranes, a physically and biochemically irregular interface that is difficult to study and manipulate. To characterize the spatial and temporal mechanisms of receptor activation, we developed a supported membrane platform that presents ephrin A1 to live cells expressing EphA2. Upon receptor-ligand binding, Eph-ephrin microclusters form, coalesce, and undergo reorganization into a multi-micron cluster that regulates protease recruitment, cytoskeletal organization, and EphA2 degradation. Using nanopatterned substrates to impose geometrically defined barriers to lateral mobility, it is possible to restrict and guide the assembly of these patterns in living cells. Measurement of this phenotype across a library of breast cancer cell lines reveals a strong correlation with the ability of a cell to invade foreign tissue. These results uncover a previously unknown mechanism of signal regulation in cancer, mediated through higher-order clustering events. Our results indicate that mechanical restriction of EphA2 pattern formation can alter the outcome of its signaling pathway, which may have consequences for certain aspects of cancer metastasis.

5. Funded Ph.D. candidate Pradeep Nair for 5 months during 2008-09
6. Funded Postdoc Hung-Jen Wu for 5 months during 2009-10
7. Submitted application entitled “Spatio-Chemical Mechanisms of EphA2 signaling in Cancer” for Department of Defense Breast Cancer Research Program Idea Award (Funding Opportunity Number W81XWH-09-BCRP-IDEA), 2009.

References:

1. D. P. Zelinski *et al.*, *Cancer Res.* **61**, 2301 (2001).
2. M. Macrae *et al.*, *Cancer Cell* **8**, 111, (2005).
3. M. Lackmann, A. W. Boyd, *Sci. Signal.* **1**, (2008).
4. P. W. Janes *et al.*, *Cell* **123**, 291 (2005).
5. J. Walker-Daniels, D. J. Riese, M. S. Kinch, *Mol. Cancer Res.* **1**, 79 (2002).

Appendix A, Meeting Abstracts:

Groves, J. T., National Institutes of Health, National Cancer Institute, Physical Sciences in Oncology National Meeting, "Physical Manipulation of EphA2 Spatial Organization Alters Cellular Response to Ephrin-A1", April 5-7, 2010, National Harbor, Maryland.

Abstract: Communication between a cell and its environment is mediated through receptors present in its membrane. Receptor activity is modulated through clustering interactions that initiate cascades of biochemical signals. This has generated a need for developing bio-patterning approaches directed at deconstructing the role of micro or nanoscale clustering in biochemical signaling. To address this challenge, synthetic nanopatterned lipid membranes presenting ephrin-A1 ligand were used to activate the EphA2 receptor tyrosine kinase in live cells. Upon ligand-receptor binding, microclusters formed, coalesced, and subsequently underwent global reorganization into a multi-micron central assembly domain. This domain regulated protease recruitment, cytoskeletal organization, and receptor turnover. Importantly, domain structure and function could be manipulated and spatially "mutated" using nanopatterns that act as lipid diffusion barriers. Quantitative measurement of nano and micro-clustering across a library of human breast cancer cell lines showed a strong correlation with the expression of a subset of genes and proteins and with reported invasion potentials. These results uncover a previously unknown mechanism of RTK signal regulation in cancer that functions solely through higher order clustering events and identify potential effector molecules which may serve as targets to treat aggressive disease.

Salaita, K., Nair, P., Gray, J., Groves, J. T., Role of Spatial Organization in Receptor Function: Eph-Ephrin Signaling, American Chemical Society Spring Meeting, March 25, 2009, Salt Lake City, UT.

Abstract: Cells respond to external cues using biochemical pathways that are typically initiated by membrane-bound receptors. The role of spatial organization of activated signal transducers is emerging as a novel concept in understanding cellular response. This has generated a need for developing biologically-relevant patterning approaches directed at understanding the role of micro- or nanoscale organization in signaling outputs. To address this challenge, a synthetic lipid membrane presenting ephrin-A1 is used to activate the EphA2 receptor. Consequently, a global, large-scale, ligand-induced receptor reorganization phenotype is identified. Nanopatterned lipid membranes are used to introduce mutations into receptor organization and this demonstrates that large-scale assemblies are functionally distinct from oligomers or microclusters. Quantitative measurement of this phenotype across a library of human breast cancer cell lines shows a strong correlation with invasion potential. Our data suggests that spatial organization of the EphA2 receptor contributes to its role in cancer progression.

Appendix B, 12-month no-cost extension application (9/1/09-8/31/10):

August 13, 2009

Monica J. Pileggi
Contract Specialist
820 Chandler Street
Fort Detrick, MD 21702-5014

RE: No-cost extension, W81XWH-08-1-0677

Dear Ms. Pileggi:

I am requesting a no-cost extension for my DA award titled "Elucidating Metastatic Behavior Through Nanopatterned Membranes." As described in Aim 1 of the statement of work, we have partially characterized the response of human breast cancer cell lines to supported lipid bilayers functionalized with ephrin-A1, and used nanopatterned supported lipid bilayers to confirm the involvement of the protease ADAM10 in regulation of EphA2 spatial organization. Furthermore, as described in Aim 2, we have characterized these responses across a library of 26 human mammary epithelial cell lines and correlated the spatial organization of EphA2-ephrin-A1 complexes with all available genomic, proteomic, and behavioral data for these cell lines. Using this approach, we have discovered a strong correlation between the ability of a cell to radially transport EphA2-ephrin-A1 complexes and its ability to invade foreign tissue, and we have determined a panel of 37 proteins and 141 mRNA transcripts that serve as surrogate biomarkers for EphA2 radial transport. We have already validated some of these biomarkers, such as the receptor-ligand pair itself and the cell adhesion protein CD44, and require additional time to continue this work due to delays obtaining some critical materials from our collaborator. Please approve a 12-month no-cost extension for this DA award.

Sincerely,

Jay T. Groves

Patricia Gates
Associate Director

Appendix C, Science Publication:

Restriction of Receptor Movement Alters Cellular Response: Physical Force Sensing by EphA2

Khalid Salaita^{1,2,3*†}, Pradeep M. Nair^{1,2,3*}, Rebecca S. Petit^{1,2,3}, Richard M. Neve^{4‡}, Debopriya Das⁴, Joe W. Gray^{4,5}, and Jay T. Groves^{1,2,3,6§}

¹Howard Hughes Medical Institute, Department of Chemistry, University of California, Berkeley, CA 94720, USA.

²Physical Biosciences Division, Lawrence Berkeley National Laboratory, Berkeley, CA 94720, USA. ³Materials Sciences Division, Lawrence Berkeley National Laboratory, Berkeley, CA 94720, USA. ⁴Life Sciences Division, Lawrence Berkeley National Laboratory, Berkeley, CA 94720, USA. ⁵Department of Laboratory Medicine and Radiation Oncology, University of California, San Francisco, CA 94143, USA. ⁶Research Center of Excellence in Mechanobiology, National University of Singapore, Singapore 117543.

*These authors contributed equally to this work.

†Present Address: Department of Chemistry, Emory University, 1515 Dickey Drive, Atlanta, GA, 30322.

‡Present address: Genentech, 1 DNA Way, South San Francisco, CA 94080, USA.

§To whom correspondence should be addressed. E-mail: jtgroves@lbl.gov

Abstract

Activation of the EphA2 receptor tyrosine kinase by ephrin-A1 ligands presented on apposed cell surfaces plays important roles in development and exhibits poorly understood functional alterations in cancer. Here, we reconstitute this intermembrane signaling geometry between live EphA2-expressing human breast cancer cells and supported membranes displaying laterally mobile ephrin-A1. Receptor-ligand binding, clustering, and subsequent lateral transport within this junction are observed. EphA2 transport can be blocked by physical barriers nanofabricated onto the underlying substrate. This physical reorganization of EphA2 alters the cellular response to ephrin-A1, as observed by changes in cytoskeleton morphology and recruitment of a disintegrin and metalloprotease 10. Quantitative analysis of receptor-ligand spatial organization across a library of 26 mammary epithelial cell lines reveals characteristic differences that strongly correlate with invasion potential. These observations reveal a mechanism for spatio-mechanical regulation of EphA2 signaling pathways.

Mammalian cells exhibit marked sensitivity to physical aspects of their environment, such as compliance (1), texture (2), and geometry (3). Tensional homeostasis between and within cells contributes to proper cell differentiation, development and ultimately survival (4). Because most cellular decision making occurs via chemical processes, understanding the coupling between physical forces and chemical signaling networks is of fundamental importance. Focal adhesions, which consist of protein assemblies organized at sites where cell-surface integrin receptors bind extracellular matrix ligands, are the most widely studied interface for tensile force transduction (5). However, the majority of membrane receptors are not associated with focal adhesions. The mechanisms (and even existence) of chemo-mechanical regulatory coupling in these systems remain largely unknown.

It is becoming clear that spatial organization of cell surface receptors can regulate associated signal transduction pathways (6-9). An important corollary is that mechanical forces acting on ligands can influence receptor spatial organization and, correspondingly, signaling (10-12). Juxtacrine signaling, in which receptor and ligand reside in apposed cell membranes, represents an important class of intercellular communication where physical restriction of ligand spatial organization and movement is evident (6, 13). Here, we reconstitute the juxtacrine signaling geometry between live cells expressing the EphA2 receptor tyrosine kinase and supported membranes displaying laterally mobile ephrin-A1 ligand.

EphA2 is implicated and functionally altered in a number of cancers. In particular, 40% of human breast cancers overexpress the receptor (14). Upon binding to natively membrane-anchored ephrin-A1, EphA2 undergoes dimerization, transphosphorylation of the cytoplasmic domains, recruitment of a molecular complex with SHC and GRB2 adaptor proteins, and subsequent activation (15). EphA2 activation stimulates the mitogen activated protein kinase (MAPK) and the phosphoinositide 3-kinase pathways and recruits the c-Cbl adaptor protein and a disintegrin and metalloprotease 10 (ADAM10), both of which regulate receptor degradation (16, 17). Freely soluble ephrin-A1 ligand binds to EphA2 but fails to trigger activation unless the ligand is chemically cross-linked (18). Despite this observation, most biological and biochemical studies of EphA2 stimulation rely on soluble variants of the ligand (14). We employ a supported membrane presentation of ephrin-A1 (Fig. 1) that reveals effects of the intrinsic intermembrane physiology on the EphA2 signaling system. This presentation system allows for precise control of membrane chemical composition and lateral organization. Molecules within the supported membrane can be confined within nanoscale corrals by physical barriers to lateral mobility that are prefabricated onto the underlying substrate (7). In the present study, the barriers restrict ephrin-A1 transport (and thus EphA2 transport in the live cell) in precisely defined ways. We refer to this type of manipulation as a *spatial mutation* (7, 19); it generates chemically identical cells that differ only by the spatial configuration of molecules within the specific signal transduction pathway under study.

A fluid supported membrane doped with 0.1% biotin-functionalized lipid was used to generate synthetic cell surfaces presenting laterally mobile ephrin-A1 (Fig. 1 and fig. S1) (20-22). The ligand density on the membrane surface was adjusted to 800 ± 200 molecules/ μm^2 (fig. S2) (23), which is comparable to the density of EphA2 receptors on the surface of a representative invasive breast cancer cell line, MDA-MB-231 (fig. S3). When these cells contact functionalized supported membranes, ephrin-A1 becomes organized into microclusters over the course of 15 min (Fig. 2, A and B). Dimerization and oligomerization of Eph receptors upon ligand stimulation is well-documented (24, 25), and higher-order clusters, such as those we observe, have been proposed to exist on the basis of crystallographic studies of the molecular interface in Eph-ephrin complexes (25, 26).

We additionally observe the microclusters to undergo inward radial transport while still bound to the supported membrane, as confirmed by live-cell fluorescence imaging and reflection interference contrast microscopy (RICM), which reveals cell-substrate contact distances (fig. S4 and Movie S1) (27). Radial transport characteristics can be quantified for a population of cells by averaging the radial distribution of ligand underneath each cell at defined time points (Fig. 2B and fig. S5). Two-color total internal reflection fluorescence microscopy (TIRFM) tracking of ephrin-A1 and enhanced green fluorescent protein (EGFP) β -actin reveals substantial co-movement between image pairs, suggesting association of the actin cytoskeleton with EphA2 clusters (fig. S6) (22). Further experiments with a Rho kinase inhibitor (detailed below) confirm that EphA2 transport is driven by actomyosin contractility.

The eight different EphA receptors and the five ephrin-A ligands are known to display some promiscuous interactions, but control experiments indicate that ephrin-A1 specifically binds EphA2 (15). First, the EphA2 receptor was highly colocalized with ephrin-A1 (fig. S7A). Additionally, when cells were pretreated with EphA2 antibodies that block the binding site for ephrin-A1, no ligand clustering or cell-surface adhesion was observed (fig. S7B). Large-scale clustering of EphA2 in live cell junctions was also observed when cells that express ephrin-A1 (ZR-75-1) and cells that express EphA2 (MDA-MB-231) were brought into contact for 30 minutes. Immunostaining of cellular junctions with antibodies specific to either ephrin-A1 or EphA2 indicated accumulation at the contact zone between cells displaying cognate receptor-ligand pairs. Such accumulations resemble those observed in cell-supported membrane experiments (Fig. 2A and fig. S8). Radial transport of receptor-ligand complex was not observed when ephrin-A1-expressing cells contacted EphA2-functionalized supported membranes (fig. S9); thus, receptor translocation is ligand-induced and driven only by the EphA2-expressing cells.

In the preceding experiments, Eph-ephrin binding provided the only physical link between the cell and the supported membrane. RICM confirmed that EphA2 – ephrin-A1 clusters colocalize with the regions of closest intermembrane contact (Fig. 2C). To determine if the observed inward radial transport may be an indirect consequence of intermembrane anchoring, a cyclic RGD (Arg-Gly-Asp) peptide-lipid conjugate was included in the supported membrane (22). This peptide serves as a binding partner for integrins on the cell surface (28), and was presented as a binary mixture with ephrin-A1 on the supported membrane in varying densities. RICM images revealed progressively larger cell – supported membrane contact areas with increasing RGD peptide density, but with no change in EphA2 organization (Fig. 2D). Immunostaining for β_1 , $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins, known markers of focal adhesions, did not show colocalization with EphA2 (fig. S10). Thus we conclude that the radial transport of EphA2 is selective and independent of integrin-mediated adhesion and signaling.

Importantly, radial transport of ligand-stimulated EphA2 is dependent on the lateral mobility of ephrin-A1 in the supported membrane. Fully saturated 1,2-dipalmitoyl-*sn*-glycero-3 phosphocholine (DPPC) lipids form a non-fluid bilayer in the gel phase at 37 °C and ephrin-A1 displayed on these membranes (22) failed to exhibit microcluster formation or inward transport upon interaction with cells (Fig. 2E). This correlated with differences in EphA2 signaling as measured by receptor phosphorylation and degradation, which are hallmarks of ligand-induced activation (16). When identical numbers of cells ($\sim 1 \times 10^5$) were plated onto fluid and non-fluid supported membranes doped with an identical density of ephrin-A1 binding sites (1:1000 biotin-DPPE), the ephrin-A1 tethered to non-fluid DPPC membranes induced $\sim 50\%$ less EphA2 degradation, and $\sim 40\%$ less tyrosine phosphorylation than did ephrin-A1 tethered to control fluid membranes (Fig. 2F).

Furthermore, on fluid membranes, ephrin-A1 clusters colocalized with the areas of highest tyrosine phosphorylation and radial transport of Eph-ephrin complexes coincided with substantial f-actin reorganization (Fig. 2C and fig. S11).

Cytoskeleton reorganization is known to result from ligand-dependent tyrosine phosphorylation of EphA2 and subsequent downstream signaling processes (29). This ultimately contributes to cell contact-dependent repulsion and tissue patterning (15). EphA2 can remodel the cytoskeleton through activation of the small guanosine triphosphatase RhoA (30), a process implicated in the high motility and invasive ability of malignant tumor cells (31). To explore the effects of this process on EphA2 transport we used the selective Rho-associated kinase inhibitor Y-27632 to block actomyosin contractility (32). MDA-MB-231 cells treated with inhibitor concentrations ranging from 1 to 50 μM exhibited a dose-dependent decrease in their capacity to transport EphA2-ephrin-A1 complexes to the center of the cell—supported membrane contact junction (Fig. 2G). This observation indicates that EphA2 transport is actively driven by actomyosin contractile forces.

To examine the functional consequences of EphA2 transport, we physically manipulated EphA2 spatial organization. Supported membranes were formed on glass substrates with various patterns of metal lines (100-nm linewidth and 10-nm height) prefabricated by electron beam lithography (22). These create barriers to lateral transport within the supported membrane without otherwise influencing mobility or altering topography (fig. S12). Lipids and membrane-tethered proteins diffuse freely, but cannot cross barriers (fig. S13) (7, 10, 33). Upon binding its supported membrane—bound ephrin-A1 ligand, the EphA2 receptor and other physically associated signaling molecules become subject to the same geometrical constraints to mobility. The approach applies physical perturbations to the living cell exclusively through specific receptor-ligand couplings and the entire ensemble of receptors is uniformly affected. A variety of non-native EphA2 spatial configurations were generated by engaging cells with patterned membranes whose grid pitches ranged from 0.5 to 20 μm (Fig. 3). Immunofluorescence imaging of cells on grid-patterned constraints reveals that the confined EphA2 clusters remain heavily phosphorylated in all cases (unrestricted, 3-, 1-, and 0.5- μm —pitch barriers). EphA2 is locally triggered irrespective of geometrical constraint (Fig 3A).

In contrast, the morphology of the f-actin exhibited two discrete states as a function of the degree of physical partitioning forced onto the EphA2 receptor pattern. Cells engaging membranes with 500 nm-pitch barriers displayed a spreading morphology, with f-actin primarily in peripheral lamellipodia. This behavior is similar to that observed in cells cultured on standard glass slides or on RGD-functionalized surfaces without ephrin (fig. S14). The actin morphology dramatically changed into an annulus immediately surrounding the EphA2-ephrin-A1 assembly when cells were exposed to substrates with grid barrier pitches of 3 μm or larger (Fig. 3A). These observed differences in f-actin morphology at identical ephrin-A1 densities indicate that physical resistance to EphA2 receptor transport can change the threshold for ephrin-A1-triggered cytoskeleton reorganization.

The recruitment of effector molecules such as phosphatases or proteases is one mechanism used to dampen EphA2 signaling levels. In particular, ADAM10, a zinc-dependent transmembrane protease, is implicated in the ectodomain trans-shedding of ephrin-As as a consequence of Eph receptor binding (17). ADAM10 has been shown to weakly associate with Eph receptors at the plasma membrane and to preferentially bind receptor-ligand complexes. Proteolytic cleavage by ADAM10 occurs at the extracellular domain of ephrin-As and is hypothesized to initiate release and endocytosis of the receptor-ligand complex (17). Disengagement of the physical tether between apposed cells is thought to play a role in the observed Eph-driven cell repulsion, rather than the cell adhesion that might be anticipated due

to strong receptor-ligand binding (14, 17). When cells were triggered with fluid ephrin-A1 for 1 hr and stained for ADAM10, we observed that ADAM10 was selectively recruited to the cell—supported membrane interface (Fig. 3B and figs. S5 and S15). However, when the EphA2 radial transport was mechanically hindered with metal grid patterns, ADAM10 recruitment was substantially reduced and selective colocalization with EphA2 was abrogated at the 60-min time point (Fig. 3B). Cross-shaped metal patterns with a similar coverage area to that of the grids (4% of surface area) still allow ephrin-A1 radial transport, and do not drastically affect ADAM10 recruitment. This confirms that ADAM10 recruitment can be regulated by physically interfering with EphA2 transport and is not simply diminished by the presence of metal patterns in the supported membrane.

To quantify ADAM10 recruitment to receptor-ligand complexes, TIRFM was used to measure cell surface EphA2 and ADAM10 levels of an identical set of cells ($n=477$ cells) that displayed a range of receptor spatial mutations. Whereas the amount of EphA2 remained constant, the amount of recruited ADAM10 decreased with the size of the observed EphA2-ephrin-A1 clusters (Fig. 3B). In addition, the colocalization of ADAM10 with EphA2 (as measured by Pearson correlation coefficient, r) also decreased. Control experiments with cross-shaped metal lines and 20- μm —pitch grids all confirm that these results are a consequence of receptor spatial organization and physical constraint. Cells cultured on two-component membranes displaying the cyclic RGD peptide along with ephrin-A1 displayed the same response to spatial mutations, confirming that this phenomenon is independent from RGD-mediated integrin adhesion and signaling (fig. S16).

These spatial mutation experiments demonstrate that physical manipulation of EphA2 – ephrin-A1 microcluster organization alters the cellular response to ephrin-A1. There are both spatial and mechanical aspects to these results. The cell applies force, via actomyosin contractility, to ligand-engaged EphA2 receptors. According to Newton's third law, grid barriers that block EphA2 transport in the spatial mutation must necessarily exert opposing forces on the receptor clusters. Spatial organization and mechanical forces are thus interconnected, resulting in an overall sensitivity of the EphA2 signaling pathway to spatio-mechanical aspects of the cellular microenvironment in which ephrin-A1 is displayed.

To investigate the generality of ligand-induced EphA2 transport beyond the MDA-MB-231 cell line, we examined a library of breast cancer cell lines. Such cell lines derived from primary tumors have been the most widely used models to elucidate how genes and signaling pathways regulate disease progression (34). When a panel of cell lines is used as a system, rather than individually, it can serve as a powerful tool to identify and investigate recurrent markers for disease progression (35). Therefore, the propensity to radially transport the EphA2 receptor was characterized in 26 cell lines (22, 34). An aliquot of $\sim 50 \times 10^3$ cells was plated onto ephrin-A1—functionalized supported membranes for 1 hour for each cell line. Live-cell fluorescence microscopy was used to image the resulting distribution of ligand under individual cells and a signature radial distribution function was determined for each cell line. Radial transport was not unique to MDA-MB-231, rather, each cell line tested displayed a distinct and characteristic degree of ligand-induced receptor reorganization (fig. S17). The diversity observed in EphA2 transport between different cell lines may result from the wide range of deregulations inherent to this library, as well as variance in EphA2 expression levels. To quantify the EphA2 radial transport phenotype, we parameterized the radial distribution functions for each cell line using linear regression, integration of area under the curve, and the ratio of peak-height to peak-width at half-maximum at time $t = 60$ min (fig. S18). These different scoring methods were robust and led to very similar values across the cell line library.

To identify the molecular signature of this spatial organization phenotype, we next performed large-scale analyses using the wealth of available data for the panel of cell lines (22). In this analysis, the measured radial transport scores serve as an unconventional *spatial biomarker* unique to each cell line and potentially associated with genomic, proteomic or phenotypic signatures in neoplasia. Invasion potentials, as measured using a modified Boyden chamber assay (34), were strongly linked (Pearson correlation $r = 0.91$, $p = 7 \times 10^{-8}$) to the receptor radial transport phenotype across the library (Fig. 4A). In contrast, EphA2 mRNA and protein expression levels did not correlate as strongly with invasion potentials, and the correlation values (r) were 0.64 and 0.53, respectively, in agreement with previous reports (34, 36). EphA2 translocation is distinct from expression, and a stronger degree of association is found between the scored receptor radial transport phenotype and invasion potentials across the breast cancer model, suggesting a link between EphA2 radial transport and tissue invasion. Additionally, a system-wide correlation of the spatial organization scores to protein and mRNA expression levels revealed 37 proteins ($p < 0.1$) and 141 mRNA transcripts ($p < 1 \times 10^{-4}$; 158 probe sets) that are associated with this phenotype (Fig. 4 B and C, and tables S1 and S2). Searches of the Kyoto Encyclopedia of Genes and Genomes and BioCarta pathway analysis databases (37) revealed that radial transport was associated with the ErbB, p53, integrin, and MAPK signaling pathways (tables S3 and S4). Notably, all of these pathways have been previously reported to associate with invasiveness and EphA2 signaling, we now show that they also associate with EphA2 spatial organization (36).

One of the proteins identified through this screen was CD44, a cell membrane-bound glycoprotein involved in cell adhesion and migration (38). The spatial organization of CD44 upon ephrin-A1 stimulation was found to anti-localize with the assembly of EphA2 (Fig. 4D), validating the involvement of CD44 in cell-driven EphA2 receptor reorganization. The system-wide correlation analysis does not necessarily provide the mechanistic details leading to EphA2 sorting; instead, it identifies proteins and genes that may serve as surrogate markers to centripetal transport.

In conclusion, we report a spatio-mechanical regulation of the EphA2 signaling pathway. Upon membrane-bound ligand stimulation, EphA2 is transported radially inwards by an actomyosin contractile process. Physical interference with this transport, which necessarily involves the imposition of opposing forces on EphA2, alters ligand-induced EphA2 activation as observed by the recruitment of the protease ADAM10 and cytoskeleton morphology. Quantitative measurement of centripetal receptor transport across a library of mammary epithelial cell lines reveals a high correlation with invasion potential and with specific gene and protein expression. These observations suggest that spatio-mechanical aspects of ephrin-A1 expressing cells and their surrounding tissue environment may functionally alter the response of EphA2 signaling systems and could play a contributing role in the onset and progression of cancer.

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Supporting Online Material

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Materials and Methods

Figs. S1 to S18

Tables S1 to S4

References

Movie S1

Figure 1

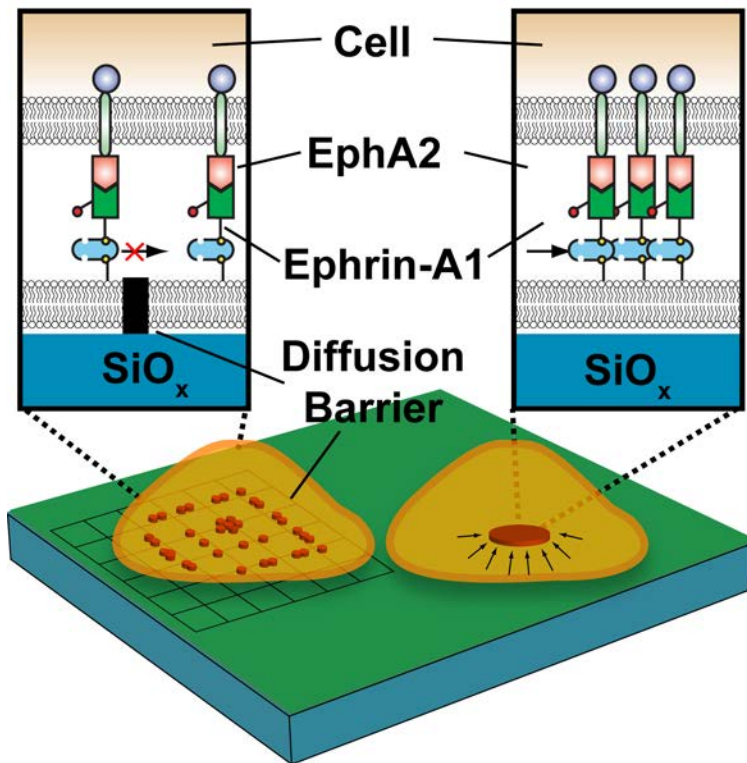


Figure 1. Scheme of the experimental platform used to trigger and manipulate the EphA2 receptor on the surface of living cells. EphA2-expressing mammary epithelial cells are cultured onto a supported membrane displaying laterally mobile, fluorescently labeled ephrin-A1 ligand. Receptors engage ligands, form clusters that coalesce and are transported to the center of the cell-supported membrane junction. Nanofabricated chromium metal lines 10 nm in height and 100 nm in linewidth (left cell) act as diffusion barriers and impede the transport of receptor-ligand complexes, leading to an accumulation of Eph-ephrin clusters at boundaries.

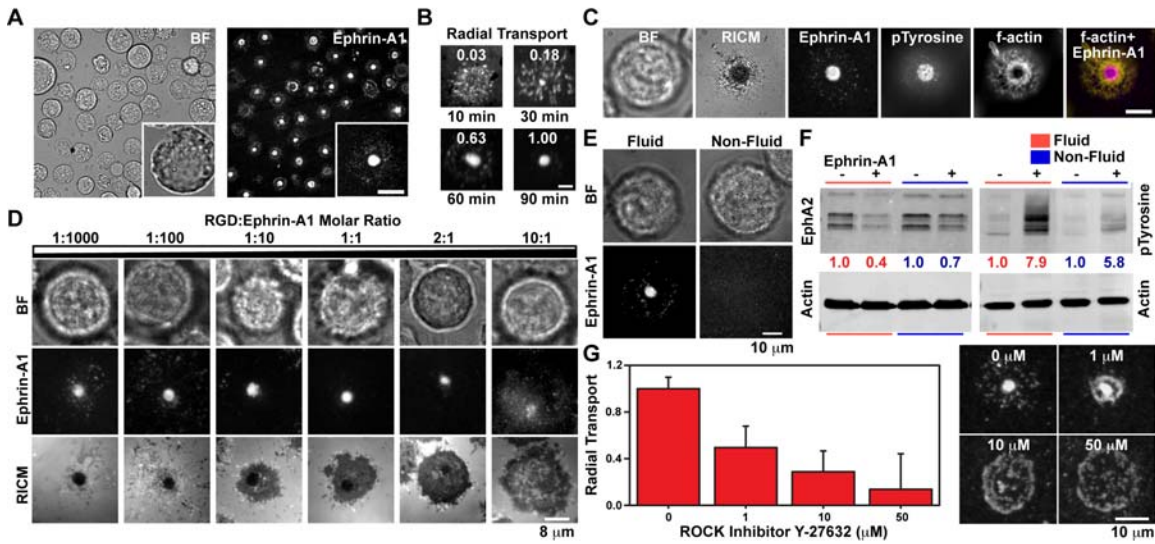
Figure 2

Figure 2. Mechanical reorganization of ligand-stimulated EphA2. **(A)** Representative bright field and epifluorescence images of MDA-MB-231 cells within 1 hr of interaction with an Alexa Fluor 647—tagged ephrin-A1—functionalized supported membrane. **(B)** Dynamics of receptor-ligand reorganization as a function of time. The radial distribution of ephrin-A1 was measured under each cell and the population average value ($n=77$ cells) is indicated above the fluorescence image for each time point. **(C)** The central EphA2 cluster is the region of highest ephrin-A1 concentration, greatest tyrosine phosphorylation and tightest cell adhesion to the substrate, and results in reorganization of the actin cytoskeleton to form a peripheral annulus. Scale bar is 5 μ m in (A) to (C). **(D)** Representative bright field, epifluorescence, and RISM images of cells 1 hour after plating on a supported membrane functionalized with binary mixtures of ephrin-A1 and cyclic RGD peptide. Ephrin-A1 and RGD were incubated in the molar ratios indicated above each panel, and show EphA2 translocation regardless of the area of the cell-supported membrane contact. **(E)** Mechanical reorganization of EphA2 requires a fluid membrane. Bilayers composed of 99.9% DPPC and 0.1% Biotin-DPPE are not fluid during cell engagement at 37 °C and, as a result, no long-range EphA2 reorganization is observed on DPPC bilayers. **(F)** Western blots of lysates collected from 1×10^5 cells cultured onto fluid and non-fluid membranes. Presentation of fluid ephrin-A1 results in more rapid and complete EphA2 activation than presentation of non-fluid ephrin-A1, as measured by EphA2 degradation and total phosphorylated tyrosine intensities. EphA2 bands are at a mass of ~100 kD. **(G)** When cells were treated with the Rho kinase inhibitor Y-27632, a dosage-dependent decrease in Eph-ephrin radial transport was observed ($n = 627$ cells), demonstrating that the cytoskeleton drives radial transport. Experiments were performed in duplicate, and radial transport was independently normalized to untreated samples from each replicate. Error bars indicate standard error for at least 139 cells at each dosage.

Figure 3

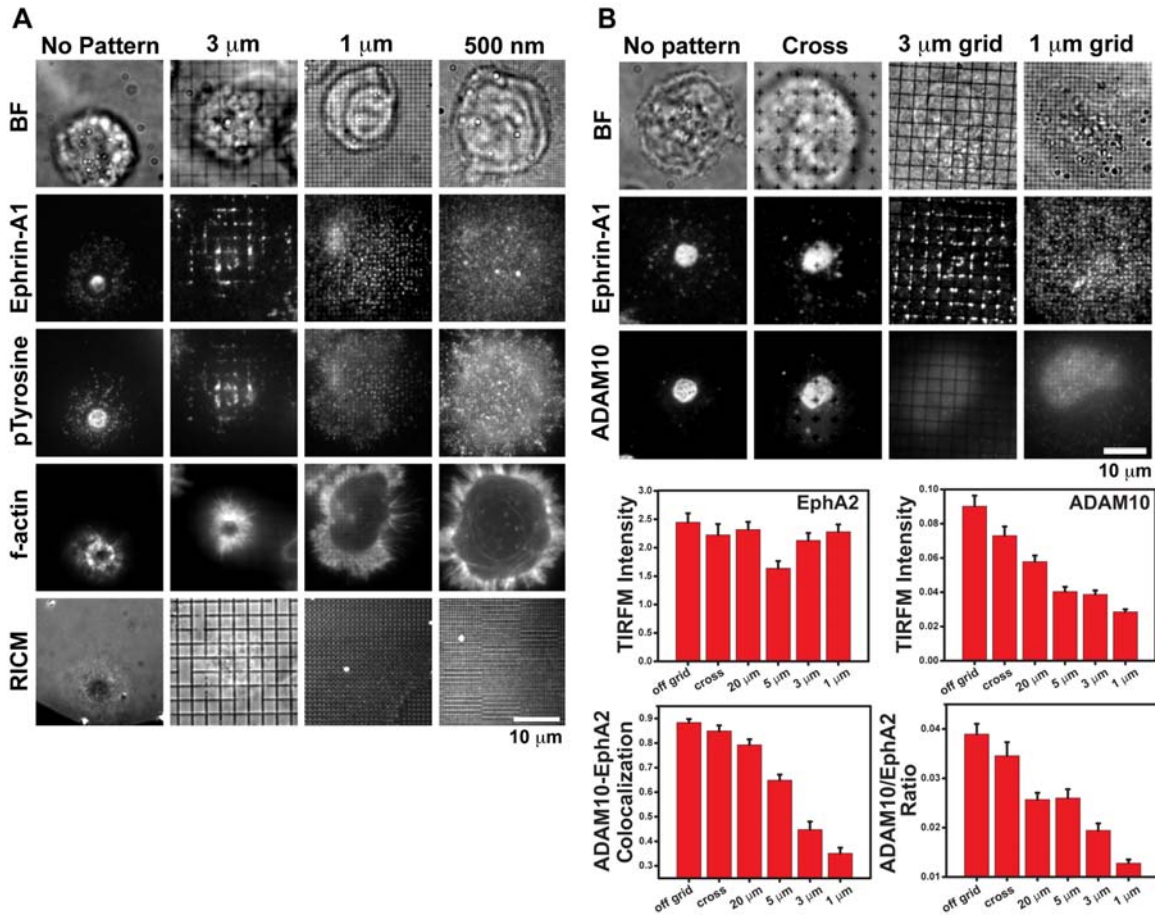


Figure 3. The functional consequences of EphA2 spatial mutation. Lateral transport of the EphA2 receptor is hindered by nanoscale chromium lines (10 nm in height and 100 nm in linewidth) prefabricated onto the glass support. MDA-MB-231 cells were allowed to engage the ephrin-A1 functionalized supported membrane for 1 hour, then they were fixed and stained for recruitment of downstream effector molecules. **(A)** Irrespective of the presence or the scale of spatial mutations, phosphorylated tyrosine colocalized with ephrin-A1. F-actin adopted an annulus peripheral to the receptor-ligand assembly when EphA2 transport was unrestricted. However, when EphA2 organization was altered, the cytoskeleton assumed a spread morphology with f-actin primarily present in peripheral lamellipodia. The spread actin morphology switched to an annulus surrounding the EphA2-ephrin-A1 assembly when cells were exposed to 3- μm —pitch barriers. **(B)** ADAM10 colocalized with the EphA2-ephrin-A1 assembly on unrestricted supported membranes. However, when EphA2 transport was restricted by metal lines on the silica substrate, the measured colocalization decreased and the ratio of ADAM10 to EphA2 also decreased ($n = 477$ cells). This indicates that mechanical restriction of EphA2 modulates ADAM10 recruitment.

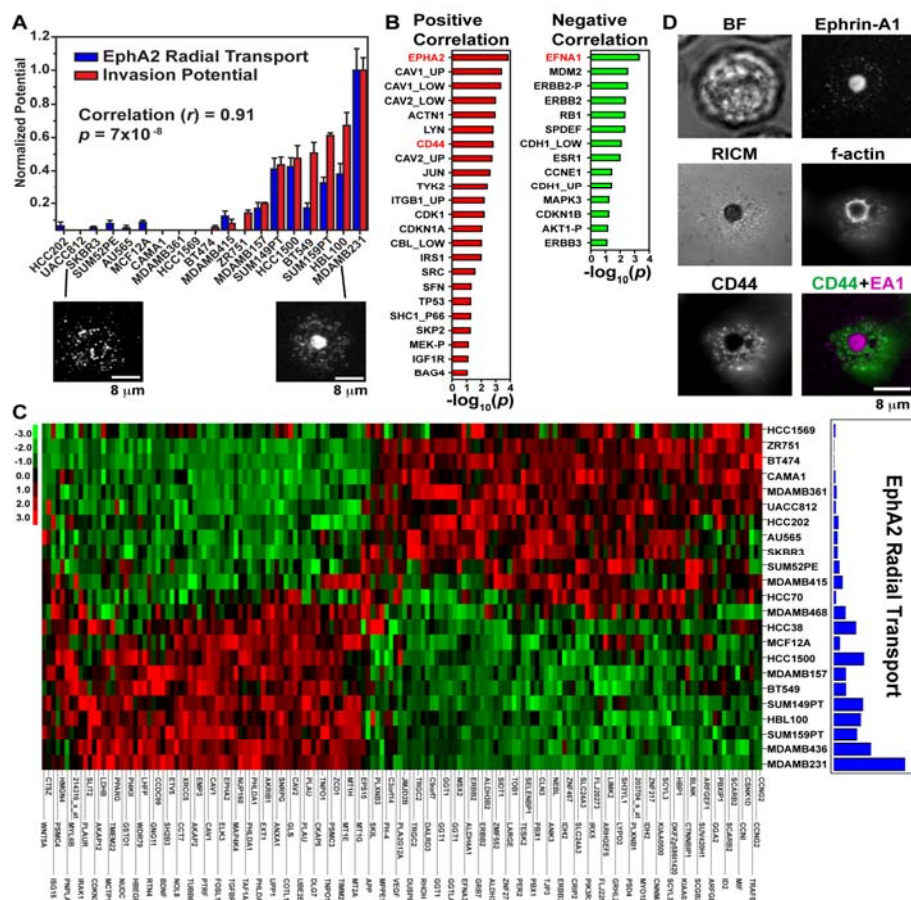


Figure 4. Correlation of EphA2 radial transport to molecular and behavior properties in breast cancer. The average ephrin-A1 ligand radial distribution functions for 26 cell lines are quantified, parameterized and then used as a spatial biomarker that is directly correlated to known biological characteristics and proteomic and genomic expression levels. **(A)** The average radial distribution function was found to exhibit a strong correlation ($r = 0.91$, $p = 7 \times 10^{-8}$) to invasion potentials, that were determined using modified Boyden chamber analysis. **(B)** The proteomic correlates ($p < 0.1$) of EphA2 radial transport are shown in the table with their associated p values, and are grouped based on the type of association (positive or negative). Proteins highlighted in red are those whose role in EphA2 reorganization has been experimentally observed. **(C)** Transcriptomic correlates ($p < 1 \times 10^{-4}$; false discovery rate $< 5 \times 10^{-3}$) of EphA2 radial transport are illustrated in a heat map. Unsupervised hierarchical clustering of expression profiles of mRNAs that are predicted to be surrogates of EphA2 radial transport show two distinct clusters of cell lines associated with the phenotype. Red indicates upregulated expression while green indicates downregulated expression. **(D)** Representative bright field, epifluorescence immunostaining, and RISM images of a cell 1 hour after plating on a supported membrane functionalized with ephrin-A1. The cell adhesion molecule CD44 was found to be significantly upregulated in protein expression in cells that underwent EphA2 radial transport. This signaling molecule was also found to be antilocalized with EphA2 upon ligand-induced activation.